## REMARKS

Claims 59, 68- 75 and 77-78 are pending.

All claims were amended in the same way, thus the support for the amendment to claims 59, 68-74, 75 and 78 is found in the specification on p.26, lines 14-18; p.26, line 27 to p.27, line 2 and FIGS. 1-3. The applicants respectfully submit that no new matter has been added. It is believed that this Amendment is fully responsive to the Office Action dated **April 3, 2006**.

The applicant appreciates the interview with the Examiner and her supervisor on July 12, 2006.

**One of the primary differences between the invention as now claimed and the prior art** is that the determination of malignancy of the thyroid tumor is not determining whether the thyroid is "**normal or malignant**", but determining whether the thyroid tumor is "**benign or malignant**."

For instance, whether a thyroid sample is normal or tumor (whether a thyroid sample is normal or abnormal) can be determined only by common practices such as X-ray examination or naked eye examination. However, it is difficult to determine **whether the thyroid tumor is benign or malignant** only by such common practices.

Therefore, the purpose of the claimed invention is to determine whether the thyroid tumor is "**benign or malignant**" (that is, **malignancy** of the thyroid tumor). So, since the present invention can determine whether the tumor thyroid is benign or malignant, it is a significant clinical advancement.

The Office Action suggests that there is no difference in the present invention and the prior art, like Tarutani, because in Figs. 1 to 3 of the present specification, there is a malignant sample of which the ratio is overlapping with the ratio of benign sample. **However, this is a misunderstanding of the point of novelty of the claimed invention.** The claimed invention is that benign and malignant can be distinguished by using the ratio of a specific thyroglobulin using the present method as explained below.

In Figs. 1 to 3, there are parts that overlap in normal or benign and malignant. However, other than at the end points, the ratios of malignant have not overlapped the ratios of benign. **That is, excluding the endpoints (end of the range), the ratio of malignant does not overlap with the ratio of benign in Figs.1 to 3.** Thus, it is possible to determine whether a sample is malignant but not benign. For instance, when the ratio of the sample is higher than the ratio of benign, the sample can positively be diagnosed malignant.

The percent amount that the ratio of malignant is overlapped with the ratio of normal or benign in each Fig. 1 to 3 of the present specification was shown in the following table.

| | Total Number | Number of data that overlapped with benign (%) | Number of data that overlapped with normal (%) |
|---|---|---|---|
| Fig.1 | 11 | 1 (9.1%) | 1 (9.1%) |
| Fig.2 | 8 | 1 (12.5%) | 3 (37.5%) |
| Fig.3 | 4 | 0 (0%) | 1 (25%) |

**As is clear from the above table, the percentage of sample of which the ratio overlapped with the ratio of benign is less than 13%, based on the data presented in the specification.** In other words, other samples of which the ratio does not overlapped with the benign can be determined as malignant but not benign.

And the ratio of benign is a ratio in the vicinity of the middle within the range of the ratio of malignant. **That is, there is no range where the sample can be determined as malignant but not benign, at all. Therefore, it is impossible to distinguish the malignant from benign by the method of Tarutani.**

**Claims 59, 68, 69, and 74 remain rejected under 35 USC 103(a) as being unpantentable over either Nakamura (US Patent 5,571,729; issued 11/5/1996) or Satomura (US Patent 5,780,247, issued 7/14/1998; effective filing 1/5/1991) in view of either Yamamoto(of record), Tarutani(of record), or Survilo(Surivilo, L.I. et al., Vestsi Akademii Navuk Belarusi, Seryya Khimichnykh Navuk, 4:103-107, 1997; abstract only).** (Office Action, p.3)

**Claims 70, 71 and 78 remain rejected under 35 USC 103(a) as being unpatentable over Katoh(US Patent 5,591,589; issued 1/7/1997) in view of either Yamamoto (of record), Tarutani (of record), or Survilo(Surivilo, L.I. et al., Vestsi Akademii Navuk Belarusi, Seryya Khimichnykh Navuk, 4:103-107, 1997; abstract only).** (Office Action, p.3)

**Claim 73 remains rejected under 35 USC 103(a) as being unpatentable over Canfield (WO/87/00289;) in view of Yamamoto (of record).** (Office Action, p.4)

**Claim 75 remains rejected under 35 USC 103(a) as being unpatentable over Katoh(supra) in view of Canfield(WO/87/00289;) and further in view of Yamamoto(supra) for the reasons of record.** (Office Action, p.4)

The same four rejections above have been maintained for the reasons explained on p.5-6 of the Office Action. In response to our previous arguments, it is commented (p.5, text lines 2-6):

> ...Yamamoto clearly compares malignant thyroids to benign and to normal on at least on pages 138 and pages 142. Yamamoto teaches that thyroglobulin isolated from **malignant** thyroid tumor tissue has a different DEAE-cellulose ion exchange elution pattern from thyroglobulin isolated from **benign and from normal** thyroids (page 138, first-2nd col.) (Emphasis added)

The rejection also cites Yamamoto for teaching about levels of sialic acid in malignant tumors.

In response to our previous arguments regarding a "double comparison" to both normal and benign, the Office Action stated (p.6, text lines 13-15):

> This is not found persuasive because all of the cited references include a comparison of lectin reactivity for thyroglobulin isolated from **malignant to lectin reactivity for thyroglobulin from benign and from normal.** (Emphasis added)

According to the Examiner on p.7, text lines 3-4 the Yamamoto teachings provide the nexus between

differential glycosylation and malignancy of thyroids.

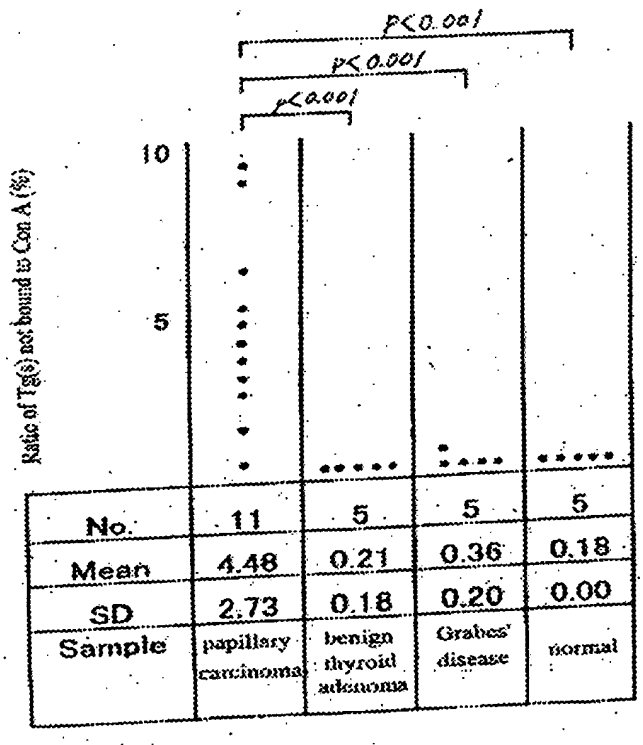
In response, the applicant has analyzed the data in the specification to make it comparable to that of the prior art, for example Tarutani, to clearly show the difference between the claimed invention and that in the prior art.

First, Figs. 1 to3 of the present specification have been prepared showing the results of statistical analysis (error analysis) of a two-sample t test (Welch's method). The results of statistical analysis are referred to by a reference (Arch Pathol Lab Med. 1998 Aug; 122(8): 715-720. A copy of the reference is enclosed. Note that the first author, Maruyama M, is one of the present inventors, and the publication date (August 1998) is later than the priority date of the present application (June 30, 1998). The left figure of Fig. 1 of the reference corresponds to Fig. 1 of the applicant's specification. The right figure of Fig. 1 of Maruyama corresponds to Fig. 2 of the present specification and Fig. 2 of Maruyama corresponds to the applicant's Fig. 3. The discussion of the Figures is on p.717, right column, line 1 to p.718, left column, line 20.

A two-sample t test (Welch's method) is used for analyzing the difference of means between two samples by using significance probability (p). The lower value of significance probability means the higher significant difference between two samples. Generally, significance probability between two samples is lower than *5%* ($p<0.05$), it is determined that there is significant difference between two samples.

Fig.l: The ratio of Tg(s) not bound to Con A (%) was 4.48 ± 2.73% (mean ± SD; n=11) for papillary carcinoma, 0.21 + 0.18% (n=5) for benign thyroid adenoma, 0.36 ± 0.20% (n=5) for Graves' disease, and 0. ±0.00% (n=5) for normal tissues. The values for papillary carcinoma tissues were significantly higher than those for Graves' disease, benign thyroid adenoma, and normal tissues ( $p<0.001$).

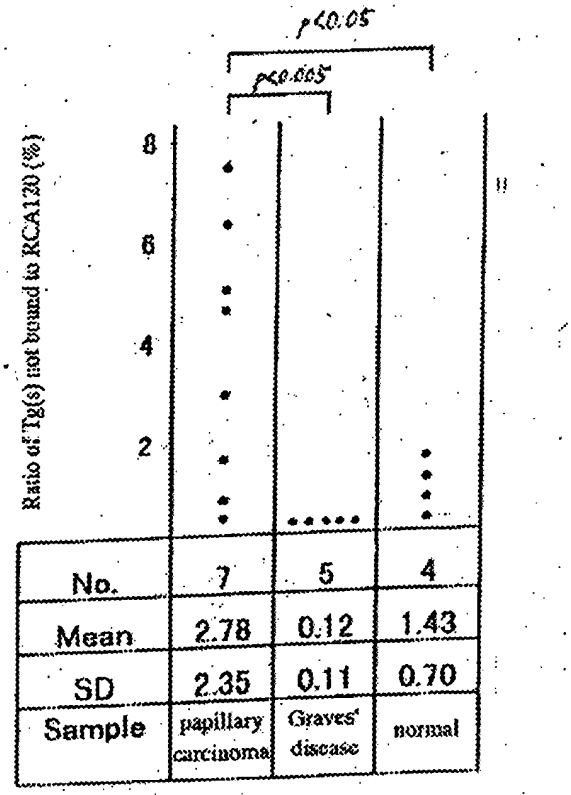
Fig. 1




| | | | | |
|---|---|---|---|---|
| No. | 11 | 5 | 5 | 5 |
| Mean | 4.48 | 0.21 | 0.36 | 0.18 |
| SD | 2.73 | 0.18 | 0.20 | 0.00 |
| Sample | papillary carcinoma | benign thyroid adenoma | Grabes' disease | normal |

Fig.2: The ratio of Tg(s) not bound to RCA120 (%) was 2.78 ± 2.35% (mean ±SD, n=7) for papillary carcinoma, 0.12 + 0.11% (n=5) for Graves' disease, and 1.43 ± 0.70% (n=4) for normal tissue. In the case of RCA-120, the differences between the ratio of Tg(s) not bound to RCA120 (%) for papillary carcinoma and Graves' disease ($p < 0.005$), as well as between papillary carcinoma and normal tissues ($p<0.05$), were statistically significant.

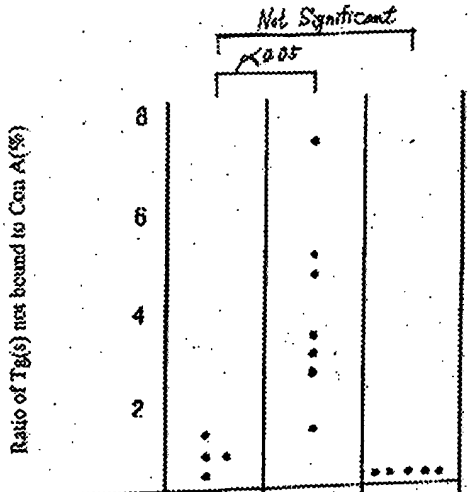
Fig. 2




| | | | |
|---|---|---|---|
| No. | 7 | 5 | 4 |
| Mean | 2.78 | 0.12 | 1.43 |
| SD | 2.35 | 0.11 | 0.70 |
| Sample | papillary carcinoma | Graves' disease | normal |

Fig.3: The ratio of Tg (s) not bound to Con A (%) was 0.83 ± 0.32% (n=4) for follicular carcinoma, 3.82 ± 2.12% (n=7) for follicular adenoma, and 0.18 ± 0.00% (n=5) for normal tissues. The values for the follicular adenoma were significantly higher than those for follicular carcinoma ($p<0.05$), however, there were no differences in unbound Tg(%) between follicular carcinoma and normal thyroid tissues.

Fig. 3

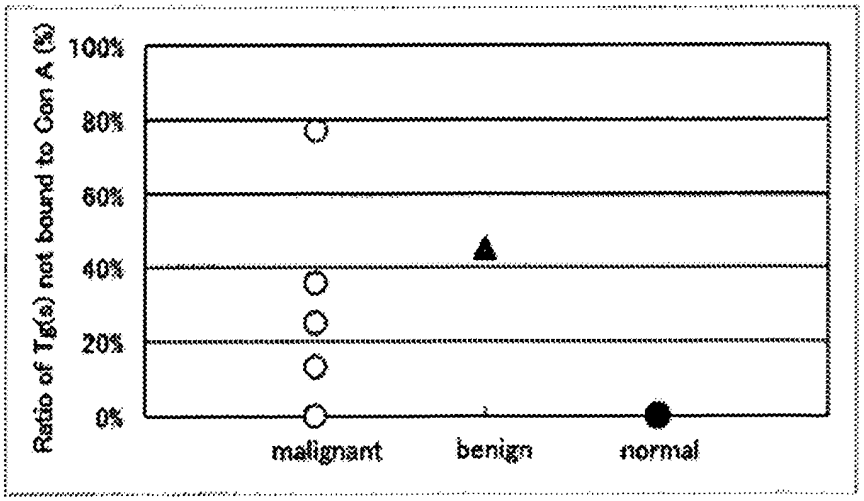



| | | | |
|---|---|---|---|
| No. | 4 | 7 | 5 |
| Mean | 0.83 | 3.82 | 0.18 |
| SD | 0.32 | 2.12 | 0.00 |
| Sample | follicular carcinoma | follicular adenoma | normal |

Thus, the analysis above proves that within a small margin or error, the applicant's claimed method is viable, whereas no such method is disclosed anywhere in the combination of prior art references. In fact, the data shown by Tarutani does not yield results according to the claimed method, as shown below.

Secondly, in order to compare the Tarutani Figure mentioned above with Fig.1 and Fig.3 of the present invention directly according to the Examiners' request, the Tarutani Figure is presented as follows: That is, No.1 of X-axis changed from 1 (normal) to malignant, No.3 of X-axis changed from 3 (malignant) to normal. Regimen of Y-axis was changed to the same with the figures of the present invention (ratio of Tg(s) not bound to Con A (%)).

(New Figure of Tarutani)



First, in Fig.1 of the present invention, the 1st column (the most left column) is related to papillary carcinoma (malignant), the 2nd and 3rd column are related to benign thyroid adenoma and Grabes' disease, respectively (benign), and the 4th column (the most right column) is related to normal. As is clear from Fig. 1, the malignant data are clearly higher than the benign data.

In Fig.3 of the present invention, the 1st column (the left column) is related to follicular carcinoma (malignant), the 2nd column (the middle column) is related to follicular adenoma (benign), and the 3rd column (the right column) is related to normal. As is clear from Fig. 3, the malignant data are clearly lower than the benign data.

**In contrast, the benign data in the middle of the range of the malignant data in new figure of Tarutani, so that malignant cannot be distinguished from benign.**

**From the above results, it is clear that malignant can distinguish from benign by the present invention, though the malignant cannot distinguish from benign by the data of Tarutani.**

The difference between the present invention and Tarutani can be explained as a difference of measuring method of the thyroglobulin.

Tarutani measures an amount of water-soluble protein using absorbance of E and the obtained amount is assumed to be the amount of Tg. The thus obtained soluble protein amount may contain Tg and other water-soluble proteins. That is, Tarutani does not quantify the Tg amount specifically. Therefore, the amount of water-soluble protein is not equal to the amount of Tg.

**On the other hand, the claimed invention measures an amount of Tg specifically by immunoassay using the anti-Tg antibodies. Therefore, the obtained amount is equal to the amount of Tg itself.**

Therefore, based on an analysis of the applicant's data compared with that shown in the prior art as well as a comparison of the methods disclosed in the prior art, the applicant asserts that reference in combination, Nakamura, Satomura, Yamamoto, Tarutani, Survilo, Katoh and Canfield cannot possibly disclose or make obvious the invention as now claimed.

It is therefore requested that the above four rejections be reconsidered and withdrawn.

**Claims 59, 68, 69, and 74 remain rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1, and 5-9 of US Patent No.5,780,247 in view of either Yamamoto (of record), Tarutani (of record) or Survilo(Surivilo, L.I. et al., Vestsi Akademii Navuk Belarusi, Seryya Khimichnykh Navuk, 4:103-107, 1997; abstract only).** (Office Action, p.7)

The applicants acknowledge this double patenting rejection and assert that a terminal disclaimer can be filed to overcome the rejection when the other claims are found allowable.

**Claims 70, 71 and 78 remain rejected under the judicially created doctrine of**

**obviousness-type double patenting as being unpatentable over claims 1 and 3 of US Patent No.5,591,589 in view of either Yamamoto (of record), Tarutani (of record) or Survilo(Surivilo, L.I. et al., Vestsi Akademii Navuk Belarusi, Seryya Khimichnykh Navuk, 4:103-107, 1997; abstract only).** (Office Action, p.8)

The applicants acknowledge this double patenting rejection and assert that a terminal disclaimer can be filed to overcome the rejection when the other claims are found allowable.

If, for any reason, it is felt that this application is not now in condition for allowance, the Examiner is requested to contact the applicants undersigned attorney at the telephone number indicated below to arrange for an interview to expedite the disposition of this case.

In the event that this paper is not timely filed, the applicants respectfully petition for an appropriate extension of time. Please charge any fees for such an extension of time and any other fees which may be due with respect to this paper, to Deposit Account No. 01-2340.

Respectfully submitted,

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PATENT TRADEMARK OFFICE

Enclosure: Maruyama et al. *A Method to Differentiate Between Thyroglobulin Derived From Normal Thyroid Tissue and From Thyroid Carcinoma Based on Analysis of Reactivity to Lectins*, Arch. Pathol. Lab. Med., Vol. 122, p.715-720, 1998.

Q:\FLOATERS\JAMIE\99\990701\990701 amend 5-22-06


# A Method to Differentiate Between Thyroglobulin Derived From Normal Thyroid Tissue and From Thyroid Carcinoma Based on Analysis of Reactivity to Lectins

*Masayuki Maruyama, MD; Ryoji Kato; Shinya Kobayashi, MD, PhD; Yoshio Kasuga, MD, PhD*

• *Objective.*—The composition of sugar chains on thyroglobulin (Tg) produced in thyroid carcinoma cells (C-Tg) is different from Tg produced in normal thyroid tissues (N-Tg). In this study, we designed a new method for detecting Tg derived from thyroid carcinoma based on the differences between C-Tg and N-Tg in the reactivity with lectins.

*Materials and Methods.*—Thyroglobulin preparations obtained from various thyroid tissues were incubated with lectins, and the amount of lectin-unbound Tg (ub-Tg) in the supernatant relative to Tg untreated with lectin was determined by enzyme-linked immunosorbent assay and expressed as ub-Tg(%). In addition, to study further the differences in glycosylation between C-Tg and N-Tg, concanavalin A binding to Tg digested with *Staphylococcus aureus* V8 protease was analyzed on nitrocellulose membrane after Western blotting.

*Results.*—The ub-Tg(%) in C-Tg from papillary carcinoma was significantly higher than in Tg from Graves' disease, benign goiter, and normal thyroid tissue for both concanavalin A and ricinus communis agglutinin-120. Concanavalin A did not appear to bind to Tg from papillary carcinoma after V8 treatment by Western blot analysis. The ub-Tg(%) in Tg from follicular adenoma was significantly higher than C-Tg from follicular carcinoma, whereas there were no differences in ub-Tg(%) between follicular carcinoma and normal thyroid tissue in concanavalin A treatment.

*Conclusions.*—These results suggest our new methods can distinguish both between C-Tg from papillary carcinoma and N-Tg, and between follicular carcinoma and follicular adenoma in thyroid tissue specimens. Thus, this type of analysis may be applicable to differentiate C-Tg from N-Tg in thyroid aspirates for the adjunctive cytodiagnosis of thyroid carcinoma.

(*Arch Pathol Lab Med.* 1998;122:715–720)

The composition of sugar chains on thyroglobulin (Tg) produced in thyroid carcinoma cells (C-Tg) is known to be different from that produced in normal thyroid tissues (N-Tg).[1–5] Miscellaneous lectins have been used to study the differences in carbohydrate structures on Tg from thyroid carcinoma and normal thyroid tissues. Tarutani and Ui[6] demonstrated that Tg from thyroid carcinoma was unretarded on concanavalin A (Con A) columns, in contrast to Tg from normal thyroid tissue. Consequently, we designed a new method for detecting Tg derived from thyroid carcinoma based on the differences of the reactivity of Tg to lectins.

## MATERIALS AND METHODS

### Subjects

Thyroid tissue specimens were obtained at surgery, and the diagnoses of the thyroid specimens were confirmed by pathologic examination as follows: 21 specimens of papillary carcinoma, 4 specimens of follicular carcinoma, 7 specimens of follicular adenoma, 5 specimens of benign goiter, 5 specimens of Graves' disease, and 15 specimens of normal tissues (areas surrounding thyroid carcinoma). Tissues were stored at −80°C until use.

### Production of Antibodies

Rabbits were injected subcutaneously with a mixture containing 50 μg of purified Tg derived from normal thyroid tissue in complete Freund's adjuvant on day 0. This was followed by three more injections on days 14, 28, and 42 in incomplete Freund's adjuvant. After test-bleed on day 56, 10 to 15 more injections with 50 μg Tg in incomplete Freund's adjuvant were administered. Sera were tested for antibody binding activity by a passive particle agglutination method for Tg (Serodia-ATG; Fujirebio Inc, Tokyo, Japan), and those sera with a titer of $10 \times 2^{10}$ or higher were selected. Anti-Tg antibodies were then further purified by filtration on a Tg-sepharose 4-B column (Pharmacia LKB Biotechnology, Bucks, England) for use in Western blot analysis.

### Preparation of Tg From Various Thyroid Tissues

A piece of frozen tissue was homogenized in phosphate-buffered saline (PBS, pH 7.5) containing 1 mmol/L phenylmethylsulfonyl fluoride (Sigma Biosciences, Dorset, England) and was centrifuged at 30000*g* for 5 minutes at 4°C. The supernatant was mixed with an equal volume of saturated ammonium sulfate for 30 minutes at 4°C, and the mixture was centrifuged at 10000*g* for 30 minutes at 4°C. Thyroglobulin-containing precipitate was dissolved in PBS and dialyzed against PBS overnight at 4°C. Macromolecular fractions were then purified by gel filtration on an ACA34 column (Pharmacia LKB) from the precipitate. The accurate concentration of Tg in these fractions was determined by the previously reported enzyme-linked immunosorbent assay for

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Tg (Tg-ELISA)[7] and adjusted to a concentration of 0.1 mg/mL for ELISA and 5 mg/mL for Con A binding.

### Preparation of Various Lectins and Sugar Solutions

The following lectins were used in this study: Con A, ricinus communis agglutinin-120 (RCA-120), and wheat-germ agglutinin (WGA), all obtained from Honen Oil Ltd, Tokyo, Japan. The lectins were diluted in PBS at concentrations of 0.1, 0.2, 0.3, 0.5, and 1 mg/mL before use. Sugars used for testing the specificity of binding of lectins with Tg were α-mannose (Man), β-galactose (Gal), *N*-acetyl-D-glucosamine (GluNAc), *N*-acetyl-D-galactosamine (GalNAc), α-fucose (Fuc), and *N*-acetylneuraminic acid (NANA), all obtained from Seikagaku Kogyo Co Ltd, Tokyo, Japan. The concentrations of sugar in solutions were 0, 0.01, 0.1, 1, 10, and 100 mg/mL.

### Treatment of Specimens With Lectins and Measurement of Tg

Fifty microliters of the partially purified Tg preparation obtained from the surgical specimens (papillary carcinoma, n = 11; follicular carcinoma, n = 4; follicular adenoma, n = 7; benign goiter, n = 5; Graves' disease, n = 5; and normal thyroid tissue, n = 5) were mixed with 50 μL of various lectin solutions and incubated at 4°C overnight. For control experiments, samples with addition of PBS only (without lectin) were incubated in the same way. The mixtures were then centrifuged at 3000*g* for 20 minutes at 4°C to remove the lectin-bound Tg. The residual unbound-Tg (ub-Tg) in the supernatant or Tg untreated with lectin in the supernatant was determined by Tg-ELISA.[7] In brief, microplates (Nalge-Nunc, Rochester, NY) were coated with 5 μg of mouse anti-thyroglobulin monoclonal antibody (TgAb) in PBS and then blocked with 1% fetal calf serum in PBS. One hundred microliters of 1% fetal calf serum in PBS followed by 50 μL of Tg preparation or standard Tg solutions were added to the wells, and the samples were incubated for 1 hour at 20°C. The plates were washed three times and then incubated for 1 hour at 20°C with a $1:10^4$ dilution of TgAb horseradish peroxidase conjugate. After washing, 100 μL of 3,3',5,5'-tetramethylbenzidine in a microwell peroxidase substrate system (Kirkegaard & Perry Labs Inc, Gaithersburg, Md) was added, and the plates were incubated for 30 minutes at 20°C. Finally, 50 μL of 1 mol/L phosphoric acid ($H_3PO_4$) was added to stop the reaction, and the optical density at 450 nm was measured. Background values for control wells without Tg, which were subtracted from test wells, were always less than 0.05 optical density units.

The ub-Tg(%) was defined as follows:

$$\text{ub-Tg(\%)} = \frac{\text{Tg Unbound to Lectin}}{\text{Tg Untreated With Lectin}} \times 100.$$

### Determination of the Optimum Amount of Lectin for Incubation With Tg

The solutions containing Tg derived from papillary carcinoma and normal thyroid tissue (one case each) were incubated with each of the three lectin (Con A, RCA-120, and WGA) solutions in mass ratio of Tg to lectin from 1:0 to 1:10. The ub-Tg(%) was determined relative to Tg not treated with lectin, which was expressed as 100%. Then, the ub-Tg ratio was calculated in each Tg:lectin ratio as follows:

$$\text{ub-Tg Ratio} = \frac{\text{ub-Tg(\%) in Carcinoma}}{\text{ub-Tg(\%) in Normal Thyroid Tissue}}.$$

### Effect of Lectins on Tg Determination in ELISA

Fifty microliters of various concentrations of the three lectins was added to wells coated with TgAb, and the wells were incubated for 1 hour. After washing, the standard Tg preparation was added, and Tg was measured as described above. Thyroglobulin concentration determined in this assay was compared with that measured by ordinary Tg-ELISA.

### Analysis of the Specificity to Tg-Lectin Binding

To examine the specificity of the reaction between the lectins (eg, Con A and RCA-120) and Tg, 50 μL of various concentrations (0, 0.01, 0.1, 1, 10, and 100 mg/mL) of six different sugars (Man, Gal, GluNAc, GalNAc, Fuc, and NANA) were added to the reaction mixture of Tg with lectin, followed by incubation as described above.

### Treatment of Tg With V8 Protease

One hundred microliters of Tg preparation (5 mg/mL) obtained from surgical specimens (papillary carcinoma, n = 10; normal thyroid tissue, n = 10) was incubated at 37°C for 1 hour with 10 μL of *Staphylococcus aureus* V8 protease (V8; Sigma) solution (20 mg/mL) in PBS; the Tg to V8 protease ratio was 25:1.

### Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli.[8] The samples were run on the 9% SDS-PAGE; molecular weight standards (Bio-Rad, Tokyo, Japan) were included on each gel. Electrophoresis was carried out for 2 hours at constant 25 mA. Western blot analysis was performed according to the method of Towbin et al.[9] The separated proteins were transferred electrophoretically onto nitrocellulose membrane for 1 hour at 150 mA and blocked for 1 hour in 1:5 diluted BlockAce (Dainihon Seiyaku Ltd, Osaka, Japan) in PBS.

### Mouse Anti-Thyroglobulin Monoclonal Antibody and Con A Binding to Tg Blotted onto Nitrocellulose Membrane and Protein Staining

The membranes were incubated with a 1:50 dilution of rabbit polyclonal Tg antibody for 1 hour at 37°C. After rinsing, the membranes were incubated for 1 hour at 37°C with horseradish peroxidase–conjugated goat anti-rabbit IgG (Kirkegaad & Perry). The Tg-TgAb complex was chemically visualized using diaminobenzidine (Nakarai Kagaku Ltd, Kyoto, Japan). Or, in separate experiments, the membranes were incubated with horseradish peroxidase–labeled Con A (Dakopatts, Glostrup, Denmark) diluted 1:300 and incubated for 1 hour at 20°C. After rinsing, the signal was chemically developed with diaminobenzidine. Furthermore, proteins transferred onto the membrane were stained with 1% Amido Black 10B (Wako Pure Chemical Industries, Osaka, Japan) for no more than 5 minutes and then rinsed in 7% acetic acid.

### Statistical Analysis

A two-sample *t* test (Welch's method) was applied for the statistical analysis.

## RESULTS

### Determination of Optimum Amount of Lectin for Incubation With Tg

In preliminary experiments, Tg preparations were incubated with lectins at different ratios (from 1:0 to 1:10), and the ub-Tg(%) in the case of papillary carcinoma ranged from 0.6% to 100% for Con A, 5% to 100% for RCA-120, and 62.4% to 100% for WGA (Table). In normal thyroid tissue, the amount of ub-Tg(%) ranged from 0.1% to 100%, 0.8% to 100%, and 91.9% to 100% for Con A, RCA-120, and WGA, respectively (Table). The highest ub-Tg ratio (63.0) between papillary carcinoma and normal thyroid tissue was found when Tg and Con A were incubated at a ratio of 1:3 (Table). For RCA-120, the best ub-Tg ratio (8.3) was found at a Tg–RCA-120 ratio of 1:3. For WGA, there was no difference between binding of lectin to Tg from papillary carcinoma and normal thyroid tissue. Consequently, in all subsequent experiments a ratio of 1:3

**Determination of the Optimum Amount of Lectin for Incubation With Thyroglobulin***

| Tg:Lectin | Con A | | RCA-120 | | WGA | |
|---|---|---|---|---|---|---|
| | Carcinoma, % | Normal Tissue, % | Carcinoma, % | Normal Tissue, % | Carcinoma, % | Normal Tissue, % |
| 1:0 | 100 | 100 | 100 | 100 | 100 | 100 |
| 1:1 | 43.5 | 1.7 | 56.7 | 42.4 | 87.1 | 97.7 |
| 1:2 | 12.5 | 0.6 | 18.0 | 8.6 | 90.1 | 100 |
| 1:3 | 6.3 | 0.1 | 7.8 | 1.1 | 83.3 | 95.3 |
| 1:5 | 0.9 | 0.2 | 5.9 | 0.9 | 85.7 | 99.9 |
| 1:10 | 0.6 | 0.1 | 5.0 | 0.8 | 62.4 | 91.9 |

* Tg indicates thyroglobulin; Con A, concanavalin A; RCA-120, ricinus communis agglutinin-120; and WGA, wheat germ agglutinin.

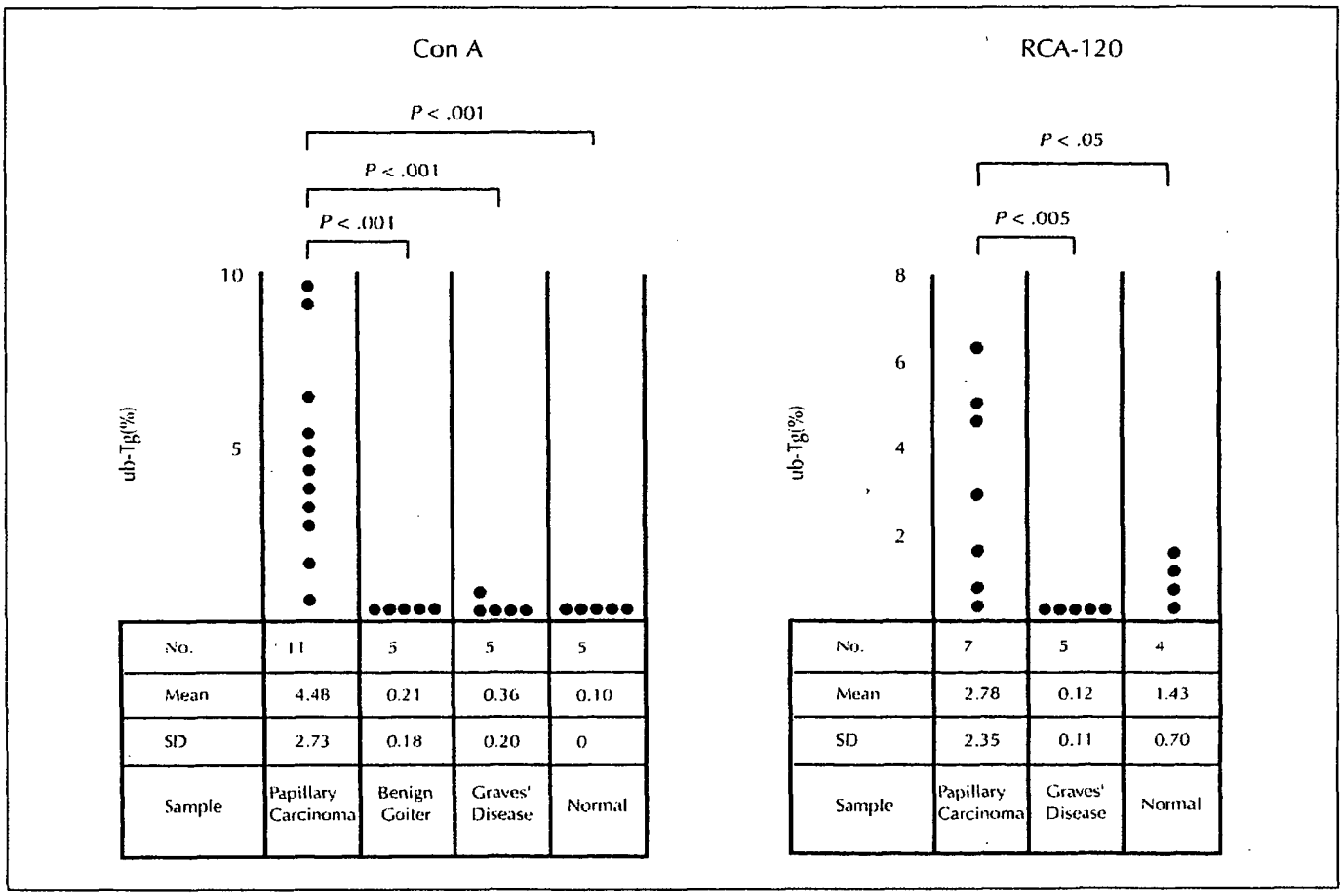


**Fig 1.**—*The ub-Tg(%) (see text for definition) of various thyroid tissues. In concanavalin A (Con A)-treated samples, the values for papillary carcinoma tissue were significantly higher than those for Graves' disease, benign goiter, and normal tissues (P < .001). In the case of ricinus communis agglutinin-120 (RCA-120), the differences between ub-Tg(%) for papillary carcinoma and Graves' disease (P < .005), as well as between papillary carcinoma and normal tissues (P < .05), were statistically significant.*

was used for incubations of Tg with Con A or RCA-120, and incubations with WGA were discontinued.

### Effect of Lectins on Tg Determination in ELISA

The three lectins (Con A, RCA-120, and WGA) had no effect on Tg determination in ELISA up to 50 mg/mL (data not shown).

### Analysis of ub-Tg(%) in Various Thyroid Tissues

Results of analysis of ub-Tg(%) in samples from papillary carcinoma, benign goiter, Graves' disease, and normal thyroid tissues are shown in Fig 1. In Con A–treated samples, the ub-Tg(%) was 4.48 ± 2.73% (mean ± SD; n = 11) for papillary carcinoma, 0.21 ± 0.18% (n = 5) for benign goiter, 0.36 ± 0.20% (n = 5) for Graves' disease, and

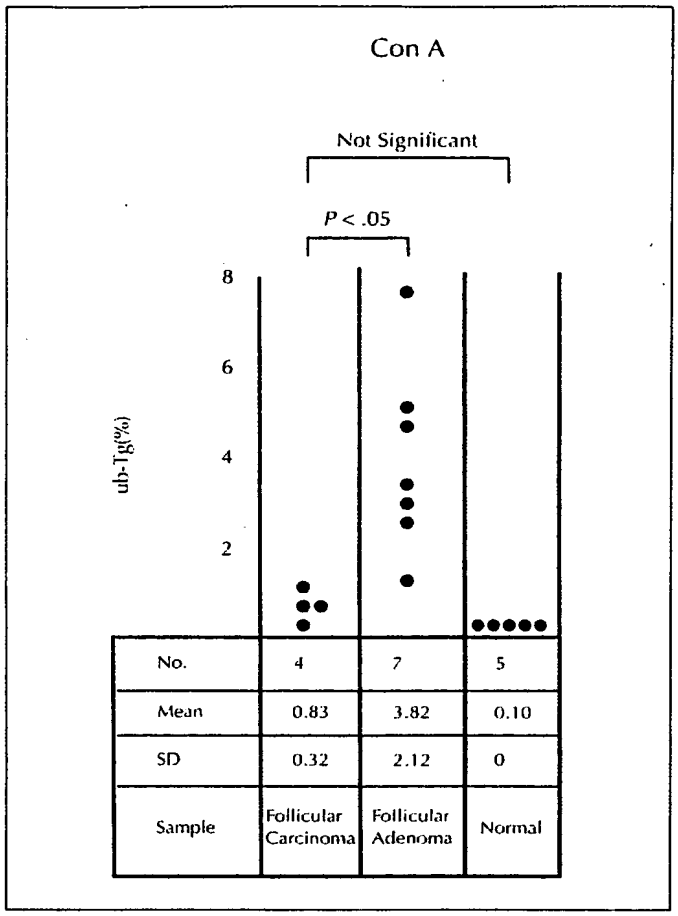


**Fig 2.**—*The ub-Tg(%) (see text for definition) of follicular carcinoma and follicular adenoma. In concanavalin A (Con A) treatment, the ub-Tg(%) values for the follicular adenoma were significantly higher than those for follicular carcinoma (P < .05).*

0.18 ± 0% (n = 5) for normal tissues. The values for papillary carcinoma tissue were significantly higher than those for Graves' disease, benign goiter, and normal tissues ($P < .001$). The ub-Tg(%) values in the RCA-120–treated group were 2.78 ± 2.35% (mean ± SD; n = 7) for papillary carcinoma, 0.12 ± 0.11% (n = 5) for Graves' disease, and 1.43 ± 0.70% (n = 4) for normal tissue. In the case of RCA-120, the differences between ub-Tg(%) for papillary carcinoma and Graves' disease ($P < .005$), as well as between papillary carcinoma and normal tissues ($P < .05$), were statistically significant.

Results of analysis of ub-Tg(%) in Con A–treated samples from follicular carcinoma and follicular adenoma are shown in Fig 2. The ub-Tg(%) was 0.83 ± 0.32% (n = 4) for follicular carcinoma and 3.82 ± 2.12% (n = 7) for follicular adenoma. The values for the follicular adenoma were significantly higher than those for follicular carcinoma ($P < .05$); however, there were no differences in ub-Tg(%) between follicular carcinoma and normal thyroid tissues.

### Analysis of the Specificity of Tg-Lectin Binding

Addition of increasing concentrations (up to 100 mg/mL) of Man to the reaction mixture of Tg and Con A resulted in the inhibition of binding of Tg to Con A (ie, ub-Tg increase) (Fig 3). The effects of NANA and GluNAc were smaller whereas Gal, GalNAc, and Fuc had no effect on Tg binding to Con A (Fig 3). Increasing concentrations of Gal (up to 100 mg/mL) inhibited binding of Tg to RCA-120 in a dose-dependent manner, whereas Man, GluNAc, NANA, GalNAc, and Fuc had no effect (Fig 3).

### Analysis of Con A Binding to Tg Blotted Onto Nitrocellulose Membrane

There were no differences between intact Tg from papillary carcinoma tissue and normal thyroid tissue in protein staining pattern, reactivity with TgAb, or Con A binding. When Tg after treatment with V8 protease was analyzed, the protein staining pattern and the reactivity with TgAb were not different for either C-Tg from papillary carcinoma tissue or N-Tg. However, Con A did not appear to bind to C-Tg after V8 treatment, whereas it reacted well with N-Tg treated in the same way (data not shown).

Similar results were obtained in each case of Tg from 10 papillary carcinomas and Tg from 10 samples of normal thyroid tissue surrounding the carcinoma.

## COMMENT

Thyroglobulin is a macromolecular glycoprotein of 660 kd with a sugar content of approximately 10%.[10–12] There are two types of sugar chains on N-Tg, namely, Unit A and Unit B. The former is characterized by high-mannose-type sugar residues[13] and the latter by mixed type.[14] In contrast, the sugar chains in C-Tg have three to five branches and are composed of complex-type sugar residues.[4] The characteristics of Tg in patients' serum and metastatic foci reflect characteristics of Tg in primary thyroid carcinoma.[15] Therefore, the ability to distinguish C-Tg from N-Tg will contribute to more accurate diagnosis of thyroid carcinoma.

The principle of the method developed in this study is based on the observation that there are differences between reactivity of sugar chains in C-Tg and N-Tg with lectins. In other words, weaker binding of lectin to sugar chains in C-Tg compared with N-Tg may be detected as an increase in the ub-Tg(%). The ub-Tg could be regarded as fractions of C-Tg that were not absorbed on lectin affinity chromatography in former studies.[2,6,16,17] In the present experiment, although Tg preparations may have contained proteins other than Tg, the Tg concentration in the solutions before and after treatment with lectins could be confirmed accurately by Tg-ELISA.

First, the effect of lectins on Tg determination in ELISA was examined. The determination of standard Tg concentrations was not affected by high concentrations of lectins (up to 50 mg/mL), therefore the presence of lectins did not interfere in the short time of reaction (1 hour) of TgAb to Tg in the first phase of Tg-ELISA.

The specificity of the reaction between the lectin and Tg was then analyzed using various concentrations of six different sugars. Concanavalin A binding was inhibited mainly by Man, but also to some extent by GluNAc and NANA (Fig 3), thus confirming Con A specificity for these types of sugar residues.[18] RCA-120 appeared to react specifically with Gal (Fig 3). There were no differences between papillary carcinoma and normal thyroid tissue in the inhibition of different lectins' binding by addition of sugars, except that slightly elevated ub-Tg(%) in C-Tg without sugars was observed. These results suggest that

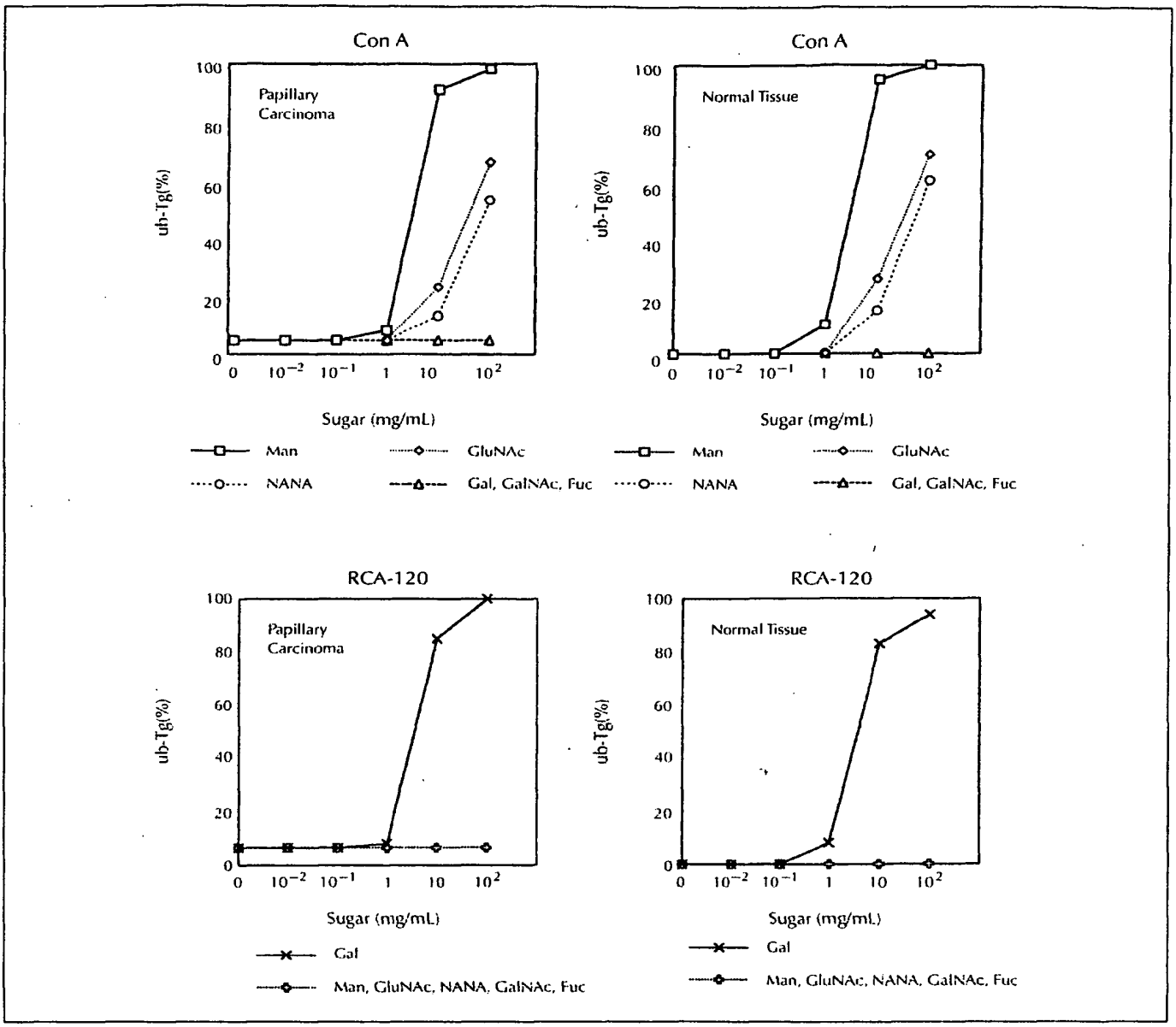


**Fig 3.**—*Effects of various concentrations of different sugars on concanavalin A (Con A) and ricinus communis agglutinin-120 (RCA-120) binding to thyroglobulin (Tg) from thyroid carcinoma and normal thyroid tissue. Addition of increasing concentrations (up to 100 mg/mL) of α-mannose (Man) to the reaction mixture of Tg and Con A resulted in the inhibition of binding of Tg to Con A. Increasing concentrations of β-galactose (Gal) (up to 100 mg/mL) inhibited binding of Tg to RCA-120 in a dose-dependent manner. GluNAc indicates N-acetyl-D-glucosamine; GalNAc, N-acetyl-D-galactosamine; Fuc, α-fucose; and NANA, N-acetylneuraminic acid.*

although sugar chains on C-Tg are similar to those on N-Tg, there may be some heterogeneity in sugar residues associated with C-Tg. This is in agreement with the findings reported by Chang,[19] who stated that the reactivity of various lectins with Tg in colloid studied by histochemical staining techniques showed heterogeneous patterns in thyroid carcinoma.

The ub-Tg(%) might depend on the reaction ratio of Tg to lectin. More binding of Tg to lectin may occur in proportion to the amount of lectin. Therefore, the optimum amount of lectin for incubation with Tg was determined as the ub-Tg ratio. The highest ub-Tg ratio (63.0) between papillary carcinoma and normal thyroid tissue was found when Tg and Con A were incubated at a 1:3 ratio (Table). In the case of RCA-120, the best ub-Tg ratio (8.3) was found at a Tg–RCA-120 ratio of 1:3.

On the basis of these preliminary experiments, ub-Tg(%) in Tg preparations obtained from various thyroid tissues was measured. The ub-Tg(%) in C-Tg from papillary carcinoma was significantly higher than that in other types of thyroid tissue with both Con A and RCA-120 (Fig 1).

In addition, to study further the differences in glycosylation between C-Tg in papillary carcinoma and N-Tg,

Con A binding to Tg digested with V8 protease (V8 Tg) was analyzed on nitrocellulose membrane after Western blotting. V8 protease, which was isolated from *Staphylococcus aureus* (V8 strain), specifically cleaves peptide linkages on the carboxyl terminal side of either asparatic acid or glutamic acid.[20] Although there were no differences in reactivity with TgAb in the cases of both intact Tg and V8 Tg, Con A did not appear to bind to Tg from papillary carcinoma after V8 treatment, whereas it reacted well with V8 Tg from normal tissue. These results suggest that the analysis of reactivity with TgAb may be of less use to distinguish C-Tg from N-Tg (despite the findings that the primary protein structure as well as the conformational structures of C-Tg are different from those of N-Tg[16]), but the examination of reactivity with Con A in Tg after treatment with V8 protease can practically contribute to the differentiation of C-Tg from N-Tg.

The differentiation of follicular carcinoma and follicular adenoma is dependent on finding capsular invasion on histopathologic examination.[21] Consequently, this distinction cannot be reliably made by cytodiagnosis using fine-needle aspiration. Therefore, our findings that the ub-Tg(%) values for follicular adenoma are significantly higher than those for follicular carcinoma may be of use in differentiating between these entities when the aspirates obtained from thyroid nodules, showing any suspicion of follicular tumors by cytodiagnosis, are tested. Although there were no differences in ub-Tg(%) for Con A between follicular carcinoma and normal thyroid tissues, examining ub-Tg(%) with different kinds of lectins may be helpful to facilitate their differentiation.

Thus, the determination of ub-Tg(%) and analysis of lectin binding to Tg digested with V8 protease (V8 Tg) should allow for the differentiation of C-Tg and N-Tg in thyroid aspirates for cytodiagnosis and consequently may be useful for the adjunctive diagnosis of thyroid carcinoma.

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## References

1. Hotta T, Ishii I, Ishihara H, Tejima S, Tarutani O, Takahashi N. Comparative study of the oligosaccharides of human thyroglobulin obtained from normal subjects and patients with various diseases. *J Appl Biochem.* 1985;7:98–103.
2. Nakajima H. Studies on the nature of thyroglobulin in human thyroid tumor tissue. *Kitakantoh Med J.* 1982;32:177–187.
3. Yamamoto K, Tuji T, Tarutani O, Osawa T. Structural changes of carbohydrate chains of human thyroglobulin accompanying malignant transformation of thyroid gland. *Eur J Biochem.* 1984;143:133–144.
4. Yamamoto K, Tuji T, Tarutani O, Osawa T. Phosphorylated high mannose-type and hybrid-type oligosaccharide chain of human thyroglobulin isolated from malignant thyroid tissue. *Biochim Biophys Acta.* 1985;838:84–91.
5. Sinadinovie J, Cvejic D, Savin S, Janic-Ziguricas M, Micic JV. Altered terminal glycosylation of thyroglobulin in papillary thyroid carcinoma. *Exp Clin Endocrinol.* 1992;100:124–128.
6. Tarutani O, Ui N. Properties of thyroglobulins from normal thyroid and thyroid tumor on a concanavalin A-sepharose column. *J Biochem.* 1985;98:851–857.
7. Kato R, Noguchi S, Noguchi A. Human serum thyroglobulin determination with monoclonal antibody one-step assay; minimum interference of autoantibody. *Endocrinol Jpn.* 1987;34:171–178.
8. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227:680–685.
9. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A.* 1979;76:4350–4354.
10. Arima T, Spiro MJ, Spiro RG. Studies on the carbohydrate units of thyroglobulin. *J Biol Chem.* 1965;240:1603–1610.
11. Spiro RG. The carbohydrate units of thyroglobulin. *J Biol Chem.* 1965;240:1603–1610.
12. Spiro MJ. Presence of a glucuronic acid–containing carbohydrate unit in human thyroglobulin. *J Biol Chem.* 1977;252:5424–5430.
13. Tsuji T, Yamamoto K, Irimura T, Osawa T. Structure of carbohydrate unit A of porcine thyroglobulin. *Biochem J.* 1981;195:691–699.
14. Yamamoto K, Tsuji T, Irimura T, Osawa T. Structure of carbohydrate unit B of porcine thyroglobulin. *Biochem J.* 1981;195:701–713.
15. Ishikita T. The existence of thyroglobulin in metastatic lymph nodes of thyroid carcinoma and significance of measurement of blood Tg level after surgery. *Folia Endocrinol.* 1995;71:105–114.
16. Tarutani O, Abe N, Hosono O, et al. Comparative studies on immunological property of thyroglobulins obtained from the thyroid tumor and the adjacent tissue. *Folia Endocrinol.* 1985;61:1176–1181.
17. Sato K. The properties of serum thyroglobulin in patients with thyroid carcinoma. *Kitakantoh Med J.* 1988;38:353–360.
18. Ogata S, Muramatsu T, Kobata A. Fractionation of glycopeptides by affinity column chromatography on concanavalin A-sepharose. *J Biochem.* 1975;78:678–696.
19. Chang Y. Histochemical study of human thyroid; with special reference to oligosaccharide recognition by lectin stains. Shinshu *Med J.* 1990;38:13–39.
20. Drapeau GR, Boily Y, Houmard J. Purification and properties of an extracellular protease of staphylococcus aureus. *J Biol Chem.* 1972;247:6720–6726.
21. Hazard JB, Kenyon R. Atypical adenoma of the thyroid. *Arch Pathol.* 1954; 58:554–563.